Carbohydrate Polymers 7 (1987) 301–312

Solution Properties of Pectin Polysaccharides. I — Aqueous Size Exclusion Chromatography of Flax Pectins

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(Received 13 January 1987; accepted 6 February 1987)

SUMMARY

Crude pectin from green flax was fractionated on Sephacryl S200 in 1 M *NaCl. The fractions obtained were characterized by measuring the anhydrogalacturonic acid (AGA) content, the degree of esterification (DE), the intrinsic viscosity* $|\eta|$ *and the molecular weight* $\bar{M}_w$. *Lower viscosities are found compared with pectins from other plant sources and a relationship exists between the DE and the molecular size of pectins. A correlation has been established between* $|\eta|$ *and* $\bar{M}_w$ *which indicates that a rather stiff conformation prevails. However the exponent of the Mark–Houwink relationship is observed to depend on the method used for clarification of solutions before measuring* $\bar{M}_w$, *due to the presence of small amount of high* $\bar{M}_w$ *particles which can considerably distort the light-scattering data witout affecting the viscosity values.*

INTRODUCTION

Pectic substances, which belong to the family of soluble dietary fibres (Selvendran, 1983), are structural heteropolysaccharides found widely in some plant primary cell walls and intercellular layers where they act as a cement between cellulosic materials. They mainly contain galacturonic acids which can be esterified by methyl groups, the extent of which (DE) will vary with age and location within the plant tissues (biosynthesis conditions) and with the method of extraction, as will the neutral sugar con-

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Carbohydrate Polymers 0144-8617/87/$03.50 — © Elsevier Applied Science Publishers Ltd, England, 1987. Printed in Great Britain

tent present in the main chain (rhamnose) and side chains (xylose, arabinose, etc.) (Pilnik & Voragen, 1970). A good knowledge of the chemical structure and solution behaviour of pectins (structure–properties relationship) is of major importance from a technological point of view and also to understand the role of pectic substances in the cell walls. This holds for pectins from fruit and vegetables in food processing as well as for those from fibre plants in the textile industry. In this regard it should be emphasized that the solution characteristics of pectin molecules have not been clearly established (as is evident from the divergent literature data relating mainly to pectins from fruit and vegetables) (Owens *et al.*, 1944; Owens *et al.*, 1946; Wollmert, 1950; Smidsröd & Haug, 1971; Berth *et al.*, 1977; Kawabata, 1977; Kawabata & Sawayama, 1977; Barth, 1980; Barbier & Thibault, 1982; Michel *et al.*, 1982; Anger & Dongowski, 1984; Fishman *et al.*, 1984; Anger & Berth, 1985; Anger & Berth, 1986). This is largely due to the number of factors (DE, charge density, neutral sugar content, presence of microgels) which could affect the molecular state in solution and therefore disturb the accurate determination of molecular weight, thus explaining the difficulty in establishing a reliable correlation between $[\eta]$ and $\bar{M}_w$ (Glikman & Orlow, 1950; Owens *et al.*, 1946; Devine, 1974; Fritsche *et al.*, 1977; Smith & Stainsby, 1977; Anger & Berth, 1985).

Despite the technical importance of flax fibres in the textile industry, little work has been done concerning the characterization of the matrix polysaccharides (pectins) found in the intercellular cement which are partially degraded during the so-called retting processes in flax.

The present work concerns the molecular characterization of pectin from the cell walls of green flax (derived during the first step of retting) by semi-preparative size exclusion chromatography (SEC) on Sephacryl S200 in 1M NaCl.

EXPERIMENTAL

Materials

Cell walls of the fibres of green flax (var. *Natasha*) were extracted by Triton X100 at 4°C for several days, purified with methanol and acetone and then oven-dried. Pectins were extracted by immersing the cell walls in hot water (90°C; 8 h). The procedure was repeated three times on the insoluble material. Filtrates were mixed, concentrated and freeze-dried. Crude pectins thus obtained had a brown coloration. Precipitation with ethanol was not used to purify crude pectins as elimination of the lower

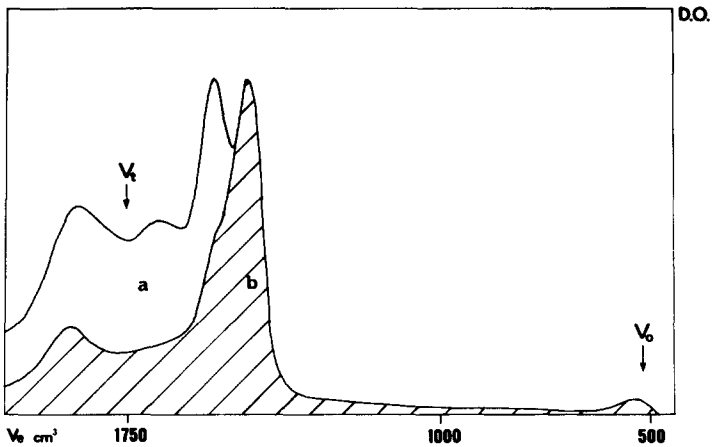

Fig. 1. Elution patterns of the flax pectin on a Sephacryl S200: (a) unrefined pectin; (b) refined by precipitation with ethanol.

$\bar{M}_w$ species has been observed to result from such a precipitation step (Fig. 1).

Semi-preparative size exclusion chromatography

Aliquots (10 ml) of 50 mg ml^{-1} crude pectin solution (in 1 M NaCl) were applied to the top of a chromatographic column (Pharmacia, 100 × 5 cm) wet-packed with Sephacryl S200 Superfine and equilibrated with dust-free 1 M NaCl. The samples were eluted with 1 M NaCl at a flow rate of approximately 3·6 ml min^{-1}. Eluate from the column was passed through a UV detector (214 nm) before collection. The column was calibrated with Dextran blue 2000 (void volume V_0) and galactose (total permeation volume V_T). The size exclusion distribution coefficient K_{av} was calculated from the relationship $K_{av} = (V_e - V_0)/(V_T - V_0)$, where V_e is the solute elution volume corresponding to the elution profile peak. Twenty-one separate injections were performed. Seven fractions corresponding to identical elution volumes V_e were collected, pooled and made salt-free by ultrafiltration (Millipore low retention volume system equipped with Pellicon membranes). An increasing loss of material was observed during ultrafiltration from fractions 1–5. A Sephadex G25 column was used to desalt fractions 6 and 7, but the attempt was not successful as the main part of the pectin material was eluted with the salt. After desalting, fractions 1–5 were concentrated and freeze-dried as the sodium salt.

Equivalent weight and esterification extent

Pectins in the H^+ form were obtained by percolation of lyophilized samples (Na^+ form) through an ion-exchange resin (H^+ form, 20–50 mesh). The free carboxylic acid content was determined by direct titration with 3×10^{-2} M KOH. De-esterification was performed in the presence of an excess of OH^- (4°C; 12 h) and the total amount of acidic groups (free and ester-type) was determined by back titration with 3×10^{-2} M HCl.

Viscosity

Viscosity measurements were made using an Ubbelhode capillary viscometer (Fica) with a solution clarified through 0·22 μm Durapore filters (Millex GV).

Light scattering

Wide-angle light-scattering measurements were made at room temperature with a Fica model 42000 photometer ($\lambda = 546$ nm) in a scattering angle range from 30° to 150°.

Low angle light-scattering measurements were performed on a laser photometer Chromatix KMX6 ($\lambda = 633$ nm) operating at low angle (4·88°), thus circumventing the extrapolation to zero angle. All solutions were purified by ultracentrifugation (100000 g) and then subjected to filtration through 0·22 μm Durapore filters prior to measurement. The refractive index increment was determined with a Brice-Phoenix differential refractometer at 546 nm ($dn/dc = 0{\cdot}155$ ml g^{-1} in 0·2 M NaCl).

RESULTS AND DISCUSSION

Size exclusion chromatography (SEC)

It is well documented that the SEC of charged macromolecules in an aqueous medium is complicated by the fact that, superimposed on the classical basic steric-exclusion mechanism, there are a number of non-steric effects which affect the elution behaviour of polar molecules: adsorption, ion exchange, ion exclusion and ion inclusion (Hamielec & Styring, 1985). Moreover, it is known that the hydrodynamic properties of polyelectrolytes are highly dependent on both the degree of ionization and the ionic strength. To minimize all these perturbing factors, the

experiments were conducted in 1 M NaCl where the effect of charges is screened.

The elution profile of the flax pectin sample (500 mg in 1 M NaCl) on Sephacryl S200 is shown in Fig. 2 where the optical density ($\lambda = 214$ nm) is plotted against V_e (and K_{av}). Two distinct elution domains exist for $K_{av} < 0{\cdot}7$ and $K_{av} > 0{\cdot}7$. To evaluate the relative areas under the various peaks, we have measured the extinction coefficients ε_{214nm} for fractions 1–5 and for the crude pectin. It is found that the extinction coefficients of the fractions are very different from one another, which explains the form of the chromatogram. From the measured ε and the corresponding chromatographic areas, the mass m_n and the percentage x of each fraction n have been calculated such that the chromatogram is now dependent only on the concentration of eluted substances. The data are reported in Table 1 and Fig. 3.

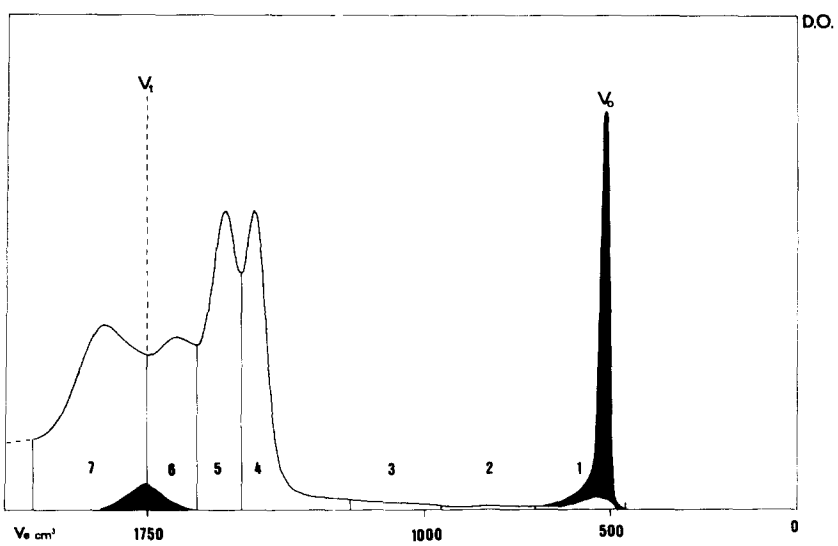

Fig. 2. Elution profile for flax pectin on a Sephacryl S200 gel in 1 M NaCl (conditions as described in the text).

It is now possible to comment on the chromatogram reported in Fig. 2. The K_{av} range $< 0{\cdot}7$ is characterized by a low optical density derived mainly from the extinction coefficient of eluted substances which represent only about 20% of the starting material. The peak located at V_0 corresponds to species which are totally excluded from the packing material. In the $0{\cdot}7 < K_{av} \leqslant 1$ range, characterized by a high UV absorp-

TABLE 1
SEC of Flax Pectin Sample

Fraction	*x (%) (apparent)*	*Area (S)*	ε_{214}	m_n *(mg)*[a]	*x (%)*[a] *(corrected)*
1	0·84	9·1	980	33	6·6
2	0·42	4·7	1300	12	2·4
3	0·9	9·7	1420	23	4·6
4	12	129·8	4625	94	18·8
5	14·4	155·7	15610	33	6·6
Rest	71·4	772·6	—	305	61
Crude Pectin *G*	100	1081·6	7280	500	100

[a] $m_n = S_n/\varepsilon_n 1$ and $x = (S_n . \varepsilon_G/(\varepsilon_n . S_G)$ where S_G and S_n are, respectively, the total and fraction areas under the chromatogram, and ε_G and ε_n the extinction coefficients of the whole pectin and its fraction *n*.

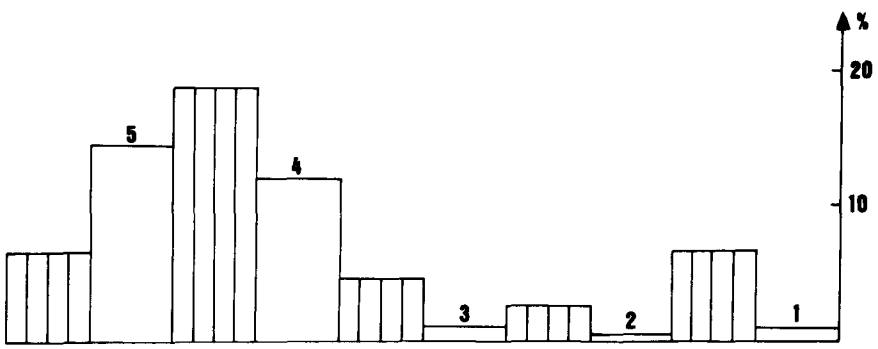

Fig. 3. Apparent (□) and actual (▥) percentages of fractions in the starting pectin.

tion, the eluate is observed to have a yellowish colour as is the case with the starting material. In the region $K_{av} > 1$ a part of the pectin material is strongly adsorbed on Sephacryl S200. As the ionic strength is high an ionic adsorption can be reasonably excluded; more probably the adsorption is of an aromatic character due to the presence of tannins of plant origin which are polyphenolic compounds. It is known that such compounds are absorbed on Sephacryl gels (Reeves *et al.*, 1970; Wasternak, 1972; Williams, 1972). The adsorbed molecules are probably low $\bar{M}_w$ species (residues of pectin chains) linked to tannin molecules. The same behaviour has been observed when fraction 7 was desalted on Sephadex G25.

Characterization of pectin fractions

Anhydrogalacturonic acid (AGA) content and degree of esterification (DE)

These parameters play an important role in the physicochemical behaviour of pectins in solution (Berth *et al.*, 1977; Rees, 1981) and can be determined separately or together by pH-metry and/or conductimetry (Thibault, 1984). The acid composition, as determined by titration, is given in Table 2.

The DE are found to lie in the range 10–40% and a relationship is observed between the acid composition and the hydrodynamic volume.

Figure 4 shows the evolution of the contents of free and ester-type carboxyl groups (calculated with M = 176 for a repeat unit of AGA)

TABLE 2
Acid Composition of Pectin Fractions

Fraction	$M_{eq}{}^{a}$	$M_{eq}{}^{b}$	*DE (%)*
1	850	515	39
2	650	450	31
3	610	440	28
4	515	385	25
5	310	280	10
G	365	325	11

[a]Before demethylation.
[b]After demethylation.

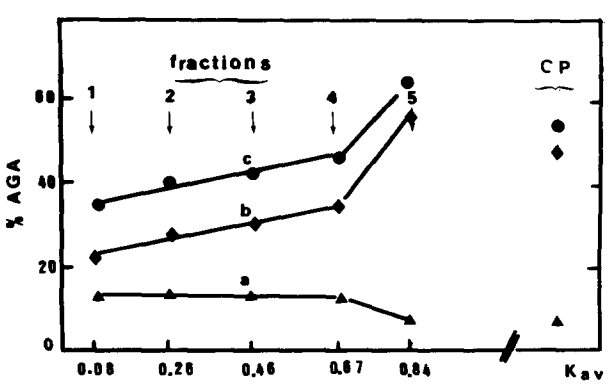

Fig. 4. Percentage of anhydrogalacturonic acid (AGA) for the flax pectins: (a) ester-type carboxyl groups; (b) free carboxylic groups; (c) total AGA.

during the elution. The total AGA increase from fractions 1–4 ($K_{av} < 0{\cdot}7$) is principally due to an increase in the AGA free extent as the ratio free/ester-type is constant in this range (12%). For $K_{av} > 0{\cdot}7$ the free AGA extent increases whereas the ester-type AGA decreases. In this exclusion range the eluted substances are probably 'oligopectins' which are more acid, i.e. short pectin chains linked to substances coming from plant cell walls (tannin molecules). It should be noted that the acid composition of flax pectins (about 40% for the four first fractions) is relatively low compared with that of pectins from other sources (Kawabata, 1977). Such a difference can be due partly to the origin of the plant material, but the method used for extraction should also be considered.

Viscosity and molecular weight

The intrinsic viscosities (in 0·2 M NaCl) of fractions 1–4 separated from the crude pectin CP are listed in Table 3.

TABLE 3
Intrinsic Viscosities of Pectins in 0·2 M NaCl

Sample	*1*	*2*	*3*	*4*	*CP*
$\lvert\eta\rvert$ ml g^{-1}	65·2	24·8	9·6	4	6·4 ($\lvert\eta'\rvert_G = 6{\cdot}1$)[a]
k'	0·46	0·75	5·8	—	5·3

[a] $\lvert\eta'\rvert_G = \sum_{i=1}^{4} \%_i^R \lvert\eta\rvert_i / 100$

It should be noted that pectins from flax are characterized by relatively low viscosities compared with pectins from other plant sources (Bock & Einsele, 1940; Kawabata, 1977). Moreover the Huggins constants k' are large for the lower viscosity samples. From the measured intrinsic viscosities of fractions 1–4 ($\lvert\eta\rvert_i$), and their actual percentage in the starting material ($\%R_i$), it is found that the contribution to the viscosity of the crude pectin ($\lvert\eta\rvert = 6{\cdot}4$ ml g^{-1}) is due mainly to the four first fractions. This means that the substances eluted for $K_{av} > 0{\cdot}7$ have insignificant dimensions, as expected for oligopectins.

The determination of $\bar{M}_w$ from light scattering gives difficulties as a result of high $\bar{M}_w$ species (microgels) which are difficult to remove, the presence of which, even in a small proportion, considerably disturbs the light-scattering data although the viscosity remains unaffected. This has been emphasized by many authors (Berth *et al.*, 1977; Smith & Stainsby,

1977; Jordan & Brant, 1978; McNeil *et al.*, 1980; Barbier & Thibault, 1982; De Vries *et al.*, 1982). The result is that large differences exist in the $|\eta| - \bar{M}_w$ relationships (Mark–Houwink plots) reported in the literature (Owens *et al.*, 1946; Anger & Berth, 1986). Figure 5 displays the Mark–Houwink plots obtained by measuring $\bar{M}_w$ at a low scattered angle (4·88°). In the same figure the results of Anger & Berth (1986) are reproduced. The comparison between curves (1) and (2) shows the influence of an ultracentrifugation step (100000 *g*; 1 h) before clarification through membrane filters (Durapore, 0·22 μm) to eliminate high $\bar{M}_w$ particles, the presence of which considerably affects the light scattering particularly at a very low angle. Moreover, the presence of such particles can be reasonably held responsible for the non-linear $|\eta| - \bar{M}_w$ rela-

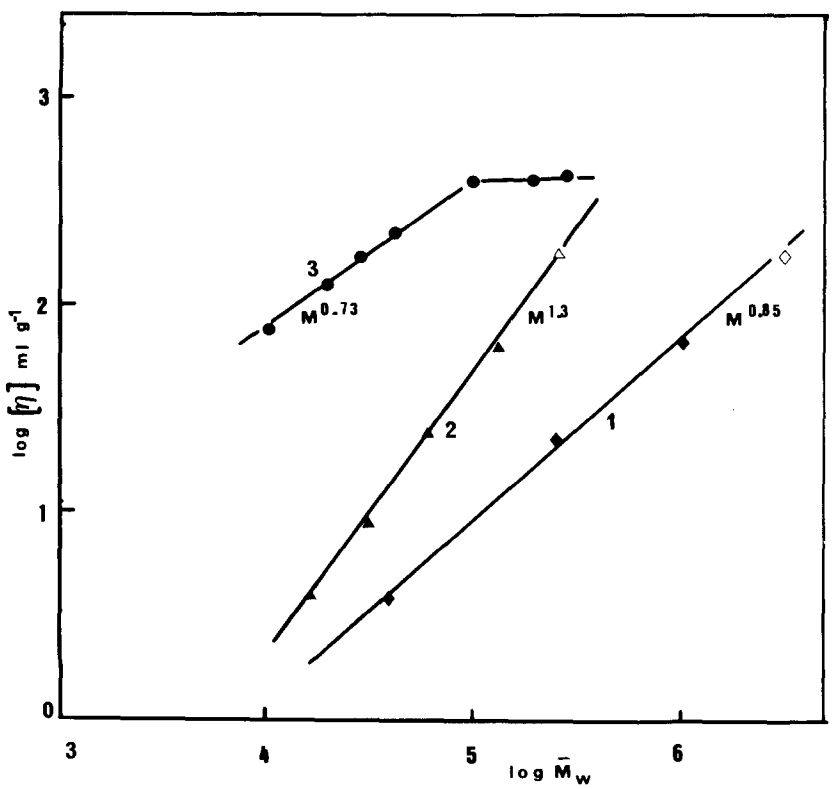

Fig. 5. Double logarithmic plots of $|\eta|$ *versus* $\bar{M}_w$ for pectins in NaCl solution: (1) filtration through 0·2 μm membrane filters; (2) ultracentrifugation (100000 g; 1 h) before filtration through 0·2 μm membrane filters; (3) repeated filtrations through membrane filters (Anger & Berth, 1986). (▲ ◆) flax pectins (DE ≈ 25–40%); (△ ◇) apple pectins (DE ≈ 45%); (●) citrus pectins (DE ≈ 58%).

tionship found by Anger & Berth (1986) above $\bar{M}_w = 10^5$ (curve 3, Fig. 5). From the data reported in Fig. 5 it is found that $|\eta|$ scales with $\bar{M}_w$ with a Mark–Houwink exponent near 0·85 (curve 1) and 1·3 (curve 2) in favour of a stiffer conformation than that reported by Anger.

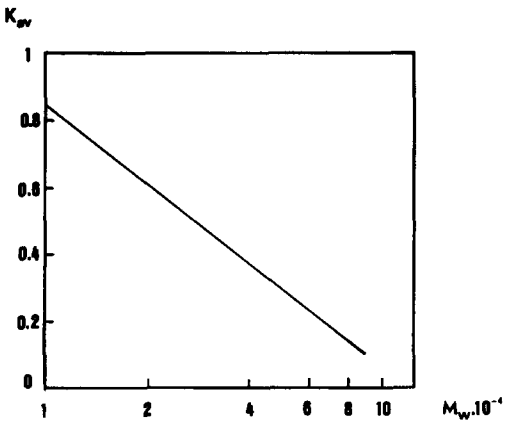

Fig. 6. Calibration plot of log $\bar{M}_w$ *versus* K_{av} for flax pectins eluted from Sephacryl S200 in 1 M NaCl.

Figure 6 shows the plot of log $\bar{M}_w$ (from curve 2, Fig. 5) against K_{av} for flax pectins on Sephacryl S200 gel. A linear relationship exists for $0 < K_{av} < 0{\cdot}7$, which covers the $\bar{M}_w$ range 10^4–10^5. From the calibration of the column it should be possible to follow changes in the pectic cement during the retting of flax.

In short it should be mentioned that, although the $|\eta| - \bar{M}_w$ relationship is particularly important to characterize the solution properties of polysaccharides as pectins, the agreement between the different authors is relatively poor. The exponent we found is in favour of a rather stiff conformation for pectin molecules from flax, which is in agreement with the lack of flexibility of the α-1,4 glycosidic bond between two galacturonic acid residues (Burton & Brant, 1969). The lower exponents in the Mark–Houwink relationships reported by authors seem not to be related to the source of pectins, as suggested by the independence on DE of the $|\eta| - \bar{M}_w$ relationship reported by some workers (Owens *et al.*, 1946; Anger & Berth, 1986), but more probably to difficulties encountered in obtaining optically clean solutions free of microgels which considerably distort the light-scattering measurements. A careful examination of all parameters which can affect the light-scattering data

should be considered. Such work is in progress and will be reported in a later paper.

ACKNOWLEDGEMENTS

This work was supported by the SCUEOR (Groupe Agro-Industrie), University of Rouen. We thank Dr C. Morvan for supplying crude pectin samples.

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